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and Retinoic Acid Receptors in Human Breast Cancer Cells

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13. ABSTRACT (Maximum 200 Words) The purpose of this research is to investigate the specific mechanisms by which transcriptional pathways may become more responsive to retinoids in cells that express estrogen receptor (ER α). Understanding how the steroid hormone receptors interact to control transcription and inhibit growth of cancer cells will suggest directions for the use of retinoids, or may provide the foundation for target-oriented therapies in breast cancer. To determine which region of the ER is required for retinoid sensitivity, several deletion mutants of ER were subcloned into a retroviral vector containing an internal ribosomal entry site (IRES) and green fluorescent protein (GFP). Target ER-negative cells were infected with virus containing ER-deletion mutant, ER-wild-type or the empty retroviral control vector. Studies with the deletion mutants indicate that the N-terminal region of ER is required to potentiate the retinoid response. Like ER-positive cells, C-terminal deletion mutants are growth inhibited by retinoids and give a greater than 100 fold induction on a β RARE compared to the retroviral control. These results indicate that the restored response to RA is mediated by the N-terminal of ER α . Blocking the AF1 domain of the ER with ICI diminishes the effect of ER, indicating that phosphorylation and activation of AF1 may play an important role in the cross-talk between ER and RAR pathways.				
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INTRODUCTION

This project explores the interaction between the expression of estrogen receptor in human breast cancer cells and their response to retinoids. It is well documented that estrogen receptor augments the growth inhibition caused by retinoids. We are currently studying why estrogen receptor positive cells respond to retinoids and, more generally, how related steroid hormones may act together to promote or inhibit cell growth. The proposed research may illuminate mechanisms by which steroid hormone receptors interact to control transcription, as well as suggest directions for the use of retinoids in the treatment of breast cancer.

ANNUAL SUMMARY

This research proposes to study the mechanism by which growth of human breast cancer cells can be inhibited by retinoids. Retinoids are derivatives of Vitamin A which are of increasing interest in the treatment of cancer patients. Retinoic acid (RA) acts by binding to a family of nuclear receptors (RAR/RXR) which in turn bind as heterodimers to response elements (RAREs) in the promoters of various genes to activate transcription. Transcription from retinoid response elements can be modulated by transcriptional intermediates which can directly stimulate or inhibit transcription in a ligand-dependent fashion. For example, in the absence of RA, SMRT and N-CoR proteins interact directly with RAR and RXR in an inhibitory manner to prevent transcription from the RAREs. Conversely, AIB1, SUG1, TIF2 and CBP are stimulatory intermediates which increase transcription in the presence of RA.

In general, ER⁺ breast cancer (ER α -positive) cells are growth inhibited by retinoids, whereas ER⁻ cells are resistant. Prior studies in this laboratory have indicated that the retinoid response observed in ER⁺ cells is independent of its ligand, estrogen, thereby suggesting an original molecular mechanism for its interaction with the retinoid response. To understand the role of the estrogen receptor, we first wished to establish comparable cell lines. For this purpose, we stably transfected a parental ER⁻ cell line (MDA-231) with the estrogen receptor. Any differences in the retinoid response when comparing these two cell lines will therefore be due to addition of estrogen receptor.

The role of the estrogen receptor in RA response is further complicated by the recent discovery of another estrogen receptor, ER β . ER β can heterodimerize with ER α , interact with the same ligands and shares greater than 90% homology with the DNA-binding domain of ER α . The level of ER β in the ER-negative and ER-positive cell lines was assessed by RNase protection assay and correlate well with the literature. ER β levels were found to be slightly higher in the ER α -negative cells MDA-MB-231 and its ER α transfected stable cell lines, while it was barely detectable in the ER α -positive cells MCF-7.

Task #1 addressed the RA-induced transcriptional activation in ER⁺ and ER⁻ cell lines. Thus far, we have tested one reporter plasmid driven by a β RARE DR5 response element (β RE-TK-CAT). The fold induction from the β RARE was greater than 100 times higher for the ER-stably transfected cell line as compared to the parental ER⁻ line. This indicates that ER modifies the transcriptional response of RAREs to RA and may be a mechanism by which ER increase the

growth inhibitory effects of RA. To confirm that ER alters the retinoid response and is not an artifact of other regulatory sites contained in the TK part of the reporter construct, transfections were also performed using a TK-CAT plasmid. The presence of ER, and addition of RA do not alter the transcription activation from the TK-CAT construct, thereby confirming that the decrease in baseline activation seen on the β RARE is specific for this promoter. Transfections will be performed with the full length RAR β promoter to evaluate the effect of ER the entire promoter,

The role of transcriptional intermediates was then evaluated in Task #2. Northern blots were performed to determine the level of expression of the inhibitory and stimulatory transcriptional intermediates. In all ER- and ER+ cell lines examined, no significant differences were found at the transcriptional levels. Although Western blots for protein levels were not yet determined due to the poor quality of antibodies available for these intermediates, the results of the Northern Blots do not indicate differences in transcriptional intermediates.

The research over this past year has focused mainly on identifying the functional domain of ER required to confer retinoid sensitivity (Task #4). For this purpose, ER- cell lines (MDA-MB-231) were stably transfected with estrogen receptor or deletion mutants. The ER wild-type and mutants were subcloned into a retroviral vector containing a packaging site, multiple cloning site, internal ribosomal entry site (IRES) and enhanced green fluorescent protein (GFP) located between two long terminal repeats (LTS). Following the subcloning step, viral producer cells were transfected with the ER-containing vector. Positively transfected cells (green fluorescent cells) were cloned and expanded to maximize the percentage of viral producers. When induced to produce virus, the stably transfected cells shed virus containing ER-GFP RNA, reverse transcriptase, integrase and protease. Media containing the virus was collected, concentrated and used to infect MDA-231 target cells. The RNA, converted to DNA by reverse transcriptase, gets integrated at random sites within the genome and a mixed population of ER (or deletion mutant)-positive green fluorescing cells is generated. The GFP does not have any deleterious effect on cellular function and provides an easy mechanism for detection of infected cells.

Our first goal was to establish an ER α (wild-type)-positive stable cell line. The presence of ER was confirmed by Northern blot but ER was undetectable by Western analysis using a C-terminal antibody. However, ER was detected by immunohistochemistry using a N-terminal antibody. Upon sequencing of the ER, this stable cell line was found to contain a base pair deletion, leading to a frameshift and a STOP codon at amino acid 346. The first stable cell line to be derived was thus labelled 231/ER₃₄₅. The ER₃₄₅ stably transfected cells survive both in stripped and full calf serum. This is in contrast to many reports in the literature which state that ER(wild-type)-reconstituted cells are growth inhibited by full calf serum and must be grown in stripped serum only. The ability of the 231/ER₃₄₅ cell line to grow in serum may be a result of its inability to bind ligand.

The growth rates of ER₃₄₅-transduced cells and the control empty-vector transduced ER-negative cells were studied to determine if the reconstitution of the ER deletion mutant restored RA sensitivity. Results from a growth curve comparing the two cell lines indicate that the ER₃₄₅-transduced cells were growth-inhibited by retinoids, whereas the parental cells were not. Since the only variation between these cell lines is the presence of ER deletion mutant, this clearly

indicates a role for the estrogen receptor in RA-mediated growth inhibition and retinoid pathways.

The transcriptional response to RA was also observed using a β RARE reporter construct. ER₃₄₅-transduced cell lines had greater fold activation on a RARE than ER-negative cell lines. This indicates that the presence of the ER deletion mutant potentiates RA-mediated transactivation. Perhaps the expression of ER in breast cancer cells may increase the expression of an intermediate that stimulates transcription in the presence of RA, or represses transcription in the absence of the RA ligand. Alternatively, ER may also bind to and recruit an inhibitory or stimulatory intermediate and thus mediate RA-responsiveness via changes in the relative expression of the above intermediates. Further deletion studies will be useful to clearly identify the functional domain of ER required to potentiate the retinoid response. The generation of these cell lines therefore provides a valuable tool for understanding the role of ER in the RA-mediated response. At the moment, virus has been collected from 4 different deletion mutants and from full length ER. The generation of stable cell lines with these deletion mutants is thus underway.

The ER C-deletion mutant stable cells lacking domain E (231/ER345) respond to retinoids as well as the ER-positive cells. Studies with the ER C-terminal deletion mutant cell line imply that the C-terminal is not required for potentiating the RA response and suggest that the effect of ER on growth inhibition and transcription is in part due to the N-terminal. The N-terminal of ER contains the activation function 1 (AF1) domain which can interact with co-regulatory molecules. We postulated that this domain may be involved in potentiating the retinoid response in ER-positive breast cancer cells by sequestering co-regulatory molecules required for basal activation of a RARE. AF1 is an important region of the N-terminal of the ER whose activity is constitutive, ligand-independent and mediates the activity of growth factors and MAP-kinase pathways. Unlike tamoxifen, which blocks only the activation function 2 (AF2) of the estrogen receptor, the anti-estrogen ICI blocks both the AF1 located in the A/B domain, and AF2, located in the E domain. In preliminary experiments, ICI increases the baseline transcription from a β RE-TK-CAT reporter construct in ER-positive cell lines, indicating that the AF1 domain may be involved in repressing the baseline activity from the β RE-TK-CAT. The increase in baseline activation due to ICI treatment in ER-positive cell lines results in a lower fold induction for the cells treated with both ICI and tRA, as compared to those treated with RA alone. In these preliminary experiments, treatment with ICI does not activate the baseline transcription of the ER-positive cell lines to the level seen in the ER-negative cell line. A dose-response curve with ICI will be performed to evaluate its effect on baseline transcription.

The MAPK pathway is involved in the phosphorylation of serine 118 in the N-terminal of ER. Preliminary studies using the MAPK inhibitors such as PD98059 (ERK inhibitor) have yielded conflicting results and as such, growth curve and transactivation experiments using SB203580 (p38 MAPK inhibitor) and PD98059 will be repeated to determine the possible role of receptor phosphorylation in potentiating the RA response.

Understanding the interaction of the signaling pathways between ER and retinoids in the suppression of cell proliferation will provide a strategic approach to the development of innovative therapies for the treatment or prevention of breast cancer. Understanding how steroid hormone receptors interact to control transcription and inhibit growth of cancer cells will suggest

directions for the use of retinoids, and hopefully for the development of novel compounds that will lead to significant advancement in the hormonal treatment of breast cancer.

Key Research Accomplishments

- Subcloned ER and ER-deletion mutants into retroviral vector.
- Generated ER-wild-type and ER-deletion mutants viral producer cell lines.
- Generated stably-transduced cell lines from the ER-negative parental line MDA-231 by infection with virus containing ER-wild-type, ER-deletion mutants and empty retroviral vector.
- Observed the transcriptional activation of ER-transduced versus control cell line on a β RARE and other reporter constructs.
- Observed the growth inhibitory effect of retinoids on the ER-transduced versus control cell lines.
- Performed Northern blots for expression of transcriptional intermediates
- Determined the ER β expression by RNase protection assay in ER-negative and ER-positive cell lines.
- Determined that the N-terminal domain of ER was important for the potentiation of the RA response.

Reportable Outcomes

- Cell lines have been developed as described above. Stably-transduced cells derived from the ER-negative parental line MDA-231 were generated by infection with virus containing ER-wild-type, ER-deletion mutants and empty retroviral vector.

- (1) Poster presentation at
- (2) The 3rd Annual Medical Science Conference between McGill University and University of Toronto, Ste-Lucie-des-Laurentides, PQ
November 5-7, 1999
"Novel Interactions Between the Estrogen and Retinoic Acid Receptor in Human Breast Cancer Cells."
- (3) DOD Era of Hope, Atlanta, Georgia
June 8-11, 2000
"Estrogen Receptor Alters the Sensitivity to Retinoic Acid in Human Breast Cancer Cells."



REPLY TO
ATTENTION OF

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